INDUCED MORPHOLOGIC VARIATION IN B. COLI

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In recent years there have appeared from time to time reports of marked morphologic changes occurring in cultures of bacteria when kept on various mediums at different temperatures. The authors of these reports have in some cases called them merely morphologic changes due to environment; in other cases the different morphologic responses were interpreted as a complex process for the perpetuation of the species. It was for the purpose of casting a little more light on this subject that the present work was undertaken.

Historical Review

The literature on the subject of this report may be briefly summarized by a description of the results obtained in the most important investigations of this character.

Almquist,[1] in 1893, reported that B. coli communior when grown in sterile sand moistened with sterile manure extract and incubated at low temperatures produced polar granules which when freed formed a new growth. When the polar granules remained attached to the cell wall they grew as coccoids.

Haslam[2] by the use of mediums containing high percentages of nitrogenous or carbohydrate material, obtained from a strain of B. coli both large rods and coccoidal forms. Cultures of both forms even after five months on these mediums reverted to the normal type when planted on standard mediums.

Adami et al[3] found that in body fluids B. coli produced stumpy bacillary forms with round chromatin bodies, like cocci, at the poles, or else large filamentous forms which broke up into the component stumpy bacilli. Abott,[4] continuing this work, reported that the minute diplococcoid forms when cultivated for some time on ascitic fluid became fixed so that they did not revert wholly to type.

Ohlmacher,[5] in working with a culture of B. coli, obtained from early autopsy, found that it produced on agar plates all gradations from diplococcoid and small streptococcoid-like individuals to long coarse filaments. Four transfers through bouillon restored it to the usual morphology. It gave the characteristic physiologic reactions, while still irregular in morphology.

Walker and Murray,[6] in testing the effect of vital staining on bacteria, discovered that B. coli produced long filaments which sometimes looked like a chain

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[1] Ztschr. f. Hyg. u. Infecktionskr. 1893, 15, p. 283.
[2] Jour. Path. & Bacteriol., 1892, 5, p. 189.
[3] Jour. Exper. Med., 1899, 4, p. 349.
[4] Jour. Path. & Bacteriol., 1900, 6, p. 326.
[5] Jour. Med. Research, 1902, 7, p. 128.
[6] Brit. Med. Jour., 1904, 2, p. 16.

of cocci when grown on a medium containing 0.2% of a saturated alcoholic solution of methyl violet. These organisms quickly regained their normal form on standard mediums.

Wilson,[7] employing a Conradi Drigalski medium containing urea, obtained thread-like forms with round or oval swellings. These swellings occurred most abundantly when the inoculum was taken from a comparatively old agar culture. The bulb-like growths were also found on the ends of the short lateral branches; they sometimes occurred free or with the remnant of a filament attached. Bacilli were found budding off from the poles of the threads or from the termination of the lateral branches. Coccoidal forms also appeared to be derived by budding from the tips of the lateral branches. These lengthened out into rod-like segments. The filaments did not persist in subcultures on standard mediums.

Hata[8] obtained thread-like organisms and globular bodies on mediums containing magnesium, calcium or sodium chloride.

Revis,[9] by growing his cultures in a broth containing increasing quantities of malachite-green up to 0.1%, discovered that two out of four of them lost the power to produce gas. This function could not be restored by repeated transfer to a medium containing sugar. Microscopic examination revealed the presence of long filaments in the culture. The growth thus produced from the typical B. coli was neither physiologically, morphologically nor culturally a colon bacillus.

Löhnis and Smith[10] made a comparative study of 42 strains[11] of bacteria by employing various mediums and making frequent microscopic examinations of both young and old cultures. They found that all the bacteria studied lived alternately in an organized and in an amorphous (symplastic) stage. "Regenerative units" appeared in the amorphous material and by germination or stretching became cells of normal shape. There was a direct union (conjunction) between two or more cells. All the bacteria multiplied not only by fission, but also by the formation of gonidia. Some of the gonidia were filtrable. The life cycle of each species of bacteria was composed of several subcycles showing wide morphologic and physiologic differences.

Almquist,[12] in his latest publication, reports that he obtained filaments and coccoidal bodies by growing 2 organisms similar to B. coli on old dry agar for 11 days, at 14 C. At 35 C., one of the cultures produced lightly and darkly stained rods, gonidia and rods in conjunction.

Hort,[13] in a further study of the morphologic variation of bacteria found, by observing the growth of a single organism on the warm stage, that binary transverse fission eventually held the field, though not absolutely, to the exclusion of other kinds of reproduction. Budding (gemmation) occurred freely in conjunction with ordinary binary fission until colonies began to form on the solid medium. This investigator[14] concludes from his latest work that the deeply staining organisms found in some cultures are types of resting cells which are able to perpetuate the strain when the environment becomes unfavorable; they

[7] Jour. Path. & Bacteriol., 1904, 11, p. 394.
[8] Centralbl. f. Bakteriol. I. O., 1908, 46, p. 289.
[9] Proc. Roy. Soc., B, 1912, 85, p. 192.
[10] Jour. Agric. Research, 1916, 6, p. 675.
[11] B. coli was not among the species studied.
[12] Svenska Läkaresällskapets Handlingar, 1917, 43, p. 543.
[13] Proc. Roy. Soc., B, 1917, 89, p. 468.
[14] Jour. Hyg., 1920, 18, p. 369.

increase in number with the age of the culture but cannot be produced by any given cultural conditions. Some cultures that do not contain these cells exhibit a lower viability than those that do.

EXPERIMENTAL WORK

Source and Treatment of Cultures.—In order to study the morphologic variation of B. coli in different environments 28 cultures of this species of bacteria were obtained from different laboratories in various parts of the country.

TABLE 1

LABORATORY NUMBERS OF ORGANISMS, NAMES GIVEN BY THE BACTERIOLOGIST WHO SENT THEM, THEIR SOURCE AND DATE OF ISOLATION

Number	Name	Source	Isolated
1	B. coli	Brownsville dysentery epidemic	1911
2	B. coli	Normal human feces	1910
3	B. coli	Medicinal spring water	1910
4	B. coli	From a bird	1901
5	B. coli	Bovine feces	
6	B. coli	Bovine feces	
7	B. coli	Human feces, D. C.	
8	B. coli	Human feces, D. C.	
9	B. coli	Human feces, Pennsylvania	
10	B. coli	Pasteur Institute	
11	B. coli	Mineral water, California	
11a	B. coli	Mineral water, California	
12	B. coli communior	Human feces	
13	B. coli	From a pigeon	
14	B. coli	From a hen	
15	B. coli	From a turtle	
16	B. coli communis	Human feces	
17	B. coli com.	Unknown	1916
18	B. coli	Bovine from intestinal feces	1916
19	B. coli	Bovine from intestinal feces	1916
20	B. coli	Human feces	1914
21	B. coli	From a case of cystitis	1911
22	B. coli	Milk, Illinois	1913
23	B. coli	Codfish feces	
24	B. coli	Pigeon feces	
25	B. coli	Y. M. C. A. swimming pool	
26	B. coli	Human feces	
27	B. coli	Oysters, Jamaica Bay, N. Y.	
28	B. coli	Soil at Veronica Spring, Calif.	

When these organisms were received they were carefully plated on beef agar and after 2 days' growth at 37 C. an inoculum was taken from the typical colon-like colonies and planted on beef agar slants. The cultures were next planted in broth and later plated on gelatin. The growth on these plates was employed as the inoculum for duplicate beef agar slants, one typical colony of each strain being selected for this purpose. These cultures were incubated for 2 days at 28 C. when one set was placed in the refrigerator for stock and the other one employed for the laboratory tests. Fresh duplicate slants were made from time to time from the stock cultures; one of the duplicates always being kept in the refrigerator for stock.

When taken from day old cultures on beef agar all except 3 of the strains were found to consist of the short rods with rounded poles that are characteristic of typical B. coli. One of these 3 contained small and medium sized rods and some threads and the other 2 were almost coccoidal in form. They all took the ordinary stains readily. All were gram-negative. None of them gave any evidence of spore formation. In day old cultures in beef broth 23 strains were motile and 6 non-motile.

One day old cultures were planted in glucose, lactose, saccharose and mannite broths. Two cultures, 4 and 8, that failed to produce gas in the glucose and lactose broths, were retained. They were tested with the B. coli in order to ascertain whether their reaction to the various mediums would show any marked differences. Eleven cultures produced gas in saccharose. The indol test showed 20 positive and 9 negative. In the duplicate tubes for nitrate reduction, 4 organisms produced a small quantity of nitrate in one set and none in the other; the remainder were good positives. The test for ammonia gave the same number of negative results. As the latter tests are not specific, they were not repeated. All except 2 cultures, 17 and 26, acidified and coagulated milk after 3 days at 37 C. Gelatine was not liquified by any of the cultures after 3 weeks' incubation at 20 C. Day old cultures on beef agar were also inoculated into Clark and Lubs' synthetic phthalate-glucose-phosphate solution for the determination of the Voges-Proskauer and the methyl red reactions. The cultures were incubated at 30 C. for 2 days and 5 days. The 2 day old cultures were used for the determination of the Voges-Proskauer reaction, and all were negative except two (22 and 23). These B. aerogenes strains were retained and tested with the coli cultures. The 5 day old cultures were employed for the methyl red test. The B. coli strains gave a P_H varying from 5.3 to 5.6. These different tests showed that 23 of the cultures were typical B. coli strains, while the remaining 6 were not. The morphology of some of the strains may be seen in fields 1 to 5 inclusive in plate 1. The rods of culture 13, field 2, lack the plumpness characteristic of B. coli cells. This strain, however, gives all the typical reactions of the species.

Methods and Mediums.—When this investigation was started a great variety of mediums were employed in order to produce a change in morphology. The cultures were found to be remarkably constant. Changes in morphology were obtained by employing unusual mediums, such as whole egg slants, egg agar, glucose egg agar, egg ammonium

lactate agar and egg starch agar, but in no case did more than 2 strains at the same temperature give the same morphologic response to a medium. As a case in point the variations with egg starch agar may be cited. Two cultures when grown for one day on this medium at 2 temperatures produced at 17 C. mostly short to medium rods, fields 8a and 9a, plate A; at 37 C. the organisms were smaller and consisted of very short rods, ovals and coccoidal forms, fields 8b and 9b, plate 1. The morphology was also studied in old cultures, but the results were not much more satisfactory in this case than when the young cultures were employed. A careful search of the stained films made from the young cultures revealed a few gonidia but, with the exception of one culture, the number found were very few.

As considerable difficulty was experienced in producing a uniformly smooth slant of whole egg after sterilization the method finally adopted is described for the benefit of those who may wish to use this or a medium producing similar difficulties. The eggs, which must be fresh, are strained 4 times through 4 thicknesses of cheesecloth. They are poured into a small suction flask and placed under a vacuum for 5 minutes with occasional shaking. About 0.75 c c is run into tubes (4″ x ½″) with a pipet and the egg then coagulated by placing the tube in a slanting position in boiling water or in an inspissator. When it is coagulated the egg is just covered with cold, distilled, sterile water and the tubes plugged and placed in a beaker containing cold water. They are sterilized in the autoclave for 30 minutes at 16 lbs. of pressure. The water over the slants may be poured off just before using. When treated in this way they will undergo this long sterilization and keep indefinitely without drying out.

When immersed in a beaker or pan of water tubes of milk may also be sterilized in the autoclave.

PLAIN EGG AGAR

Whole egg	2 c c
Agar 1.5%	7 c c

Fresh eggs must be used. Strain the eggs twice through four thicknesses of cheesecloth. Mix well with agar. Sterilize at 12 lbs. for 15 minutes on a slant stick or glass rod.

GLUCOSE EGG AGAR

Whole egg	2 c c
Agar 1.5%, Glucose 1.0%	7 c c

See directions for plain egg agar.

AMMONIUM LACTATE AGAR

Na_2HPO_4	2.0 gm.
Agar	15.0 gm.
Distilled water	1000 c c

Titrate to plus 3. Then add

$NH_4C_3H_5O_3$	10 c c
$CaCO_3$	10 gm.

Prepare the egg medium by adding 2 c c of whole egg, carefully strained, to 7 c c of this medium. Tube and sterilize at 12 lbs. of pressure for 15 minutes.

EGG STARCH AGAR

The egg starch agar is made from whole egg prepared as described in the foregoing and then 2 c c of this product are added to 7 c c of a medium containing 1.5% agar in distilled water and 0.1% soluble starch. It is slanted and sterilized in the same way as the plain egg agar.

Microscopic Study of the Living Organisms.—As the regular cultural methods produced no satisfactory results, a microscopic study of the living organisms was undertaken.

A cell was perfected in which these bacteria could grow satisfactorily and be kept under observation for a long time. It was prepared by cutting 2 rings of mechanical sheet packing about 20 mm. in outer diameter and 15 mm. in inner diameter. The packing material contained Para rubber, plantation rubber (smoked sheet) whiting, asbestos pulp, barytes, litharge, black lead talc and sulphur. Cork cutters were employed for the cutting of rings. The material used in this investigation was 1 mm. thick. One of these rings was fixed to the middle of the slide with Canada balsam and the second one then secured on top of it with the same adhesive. A third ring with an outer diameter of 13 mm. and an inner one of 9 mm. was then concentrically fastened to a clean cover glass of about the same diameter, 20 mm., as the outer edge of the rings on the slide; fig. 1 shows the structure of the cell. A is the slide, B the rings of mechanical sheet packing, C the small cover glass, D the small sealed cell, E the large cover glass and F the plasticine supports.

The slides and cover glasses were not used until the following day so that the balsam would have time to set. When cultures were to be made the cover glass with its ring was first sterilized by passing through a flame and then setting it in a sterile Petri dish. The slide with its rings was sterilized in the same way and put in the same Petri dish. A small quantity of sterile petrolatum was then spread around each ring with a sterile scalpel. The culture was prepared by smearing the inoculum on the cover glass within the ring, adding 2 or 3 drops of sterile broth from a pipet and then pressing down on the ring a small sterile cover glass of about the same diameter as the ring. There was thus formed a small sealed cell completely filled with solution. The larger cell on the slide was filled with sterile solution and the cover glass with its closed cell pressed into position. A double cell was made so that it would form a better protection against loss of solution. A strip of plasticine about 2 mm. higher than the cell was placed at each end of the slide so that when it was inverted and allowed to rest on

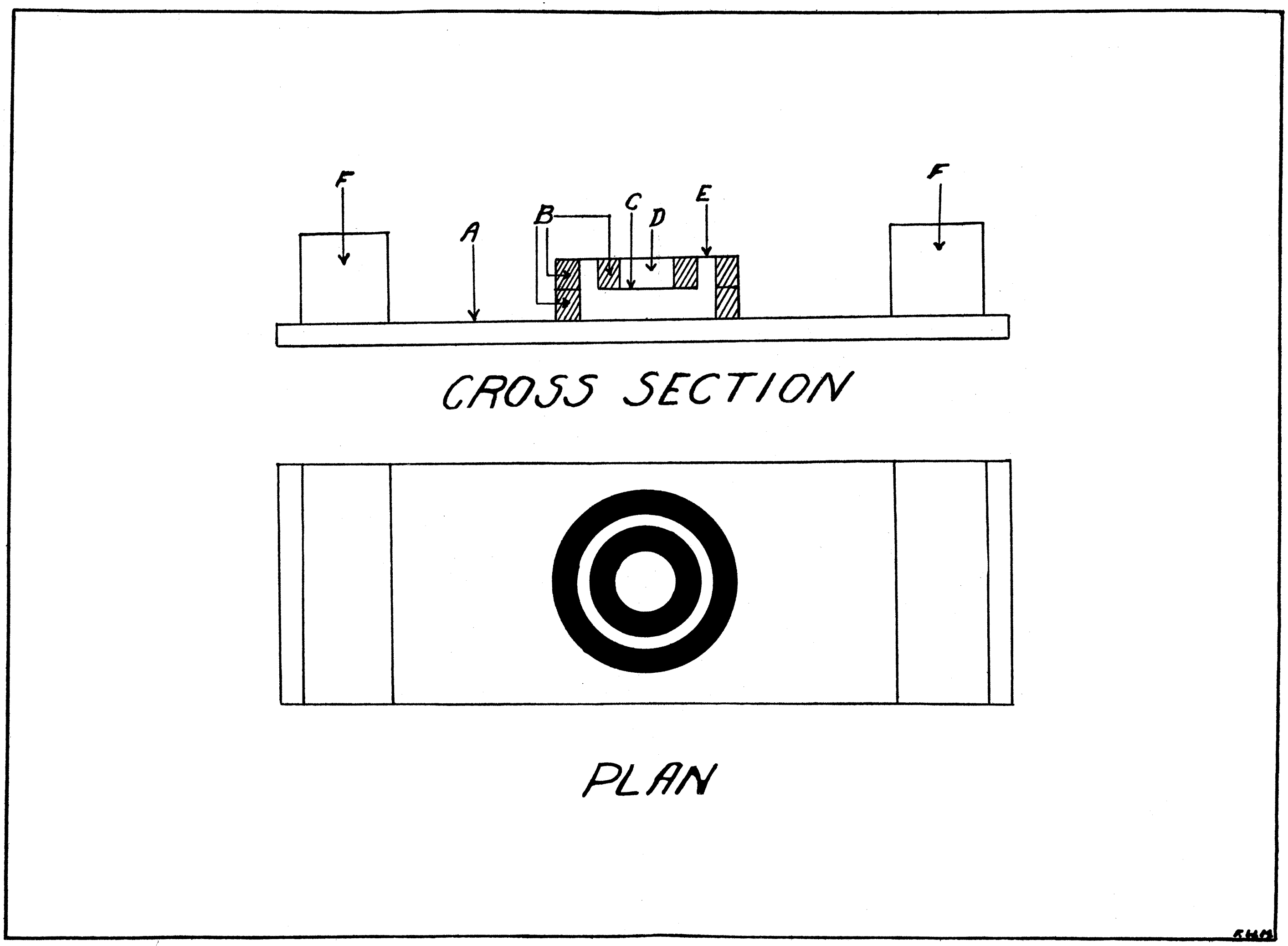


Fig. 1.—Structure of cell.

the plasticine the cell would be well clear of the surface below. The slide was incubated in a sterile Petri dish in an inverted position so that the organisms that settled from the solution would rest on the cover glass and be easily examined microscopically.

A modification of this type of cell that was of service was one in which the ring fastened to the cover glass consisted of one thickness of waxed paper. The advantage of this cell was that with the oil immersion the observer could look through the whole depth of the solution. The entire culture with its organisms could thus be examined. This cell was only serviceable when there was no gas production for the pressure of gas invariably forced the inner cover glass off.

The medium that gave the most favorable results with B. coli was an ammonium citrate broth made as follows:

KH_2PO_4 (neutral to phenolphthalein with $\frac{n}{20}$ NaOH	0.2 gm.
NaCl	0.2 gm.
$MgSO_4$	0 2 gm.
$CaCO_3$	excess
$FeCl_3$	trace
Ammonium citrate	1.0 gm.
Glucose	1.5 gm.
Distilled water	1000 c c

Filter clear. Tube and autoclave at 15 lbs. of pressure for 15 minutes.

Many cultures were examined in this cell but 10 was the only one that gave results of particular interest. It produces gonidia regularly on all mediums and more abundantly in 1-3 day old cultures than in older ones. These bodies, which are nearly always polar, are at first visible as a minute spherical cell separated from the parent by a constriction in the cell wall. They grow in this close connection until they have attained the diameter of the mother cell when the constriction is gradually elongated, principally, it would seem, by the active movement of the small cell. The constriction may attain a length of 1 micron. The whiplike snapping movements of the polar body finally break the connection, and the gonidium becomes an actively motile cell. The gonidium is much more active than the large organisms and on account of the rapidity of its movement and small size there was no opportunity to make a continuous observation of its growth after separation from the parent cell.

Indirect evidence of the growth of gonidia was obtained from the general appearance of these cultures and also aerobic ones which, during

the first 3 days, usually show a considerable number of gonidia, but as the cultures age the number of gonidia becomes less and oval forms and short rods more numerous.

A contact preparation from a colony on beef agar, field 24, plate 2, shows organisms with polar gonidia; some of the rods are almost wholly divided into small living units. The culture that produces gonidia most abundantly also usually contains more rods sticking together than the other cultures do. This adhesion of two or more cells Lohnis and Smith [10] have called conjunction. Fields 10 and 13, plate 1, show cells so joined.

When this culture, 10, is planted in an ammonium citrate broth in the cell described and incubated at 37 C., there is at first only the normal growth, but after 6 or 7 days a marked difference in the cells becomes evident. The gonidia on the cover glass or the buds still attached to the mother cell are, after this time, an opaque black while the rod remains vitreous. Some of the very short, thick rods in this culture may also become entirely black, but the long, thin rods characteristically occur either with black granular contents or with no granules. The granules are not found in any regular arrangement in the different rods. They first appear brown and finally black. This blackening was produced by the chromatin absorbing some substance which was dissolved out of the packing material. This substance occurred in a pale yellow fluid, which spread over the cover glass, and it affected only those organisms that settled in the field. The bacteria with black granules did not occur in the solution deeper than 32 microns from the cover glass. In order to determine whether this blackening of the gonidia and chromatin granules could be traced to the presence of any one particular constituent of the packing material each of its ingredients was tested in a glass cell, but in no instance did the organisms give a positive reaction.

The different absorptive power of a gonidium and a mother cell indicates that when a gonidium is formed the process is not similar to fission, except possibly in very young cells, but that there is protoplasmic differentiation whereby a nucleus is formed for the gonidium, either by the growth or the swarming of nuclear material. The important fact is that there is a difference in the contents of the two cells in that the more active nuclear material seems to fill completely the younger cell. The accumulation of the black material in the cells is a gradual process and is a measure of the activity of the cells. It

eventually results in their death. The blackening of the small cells cannot be attributed to the permeability of the cell wall because the granules in the long rods become black like the gonidia.

The black nuclei were often found near the poles of the large rods. If there was a black bud attached to the pole there was usually no black nucleus at that pole, but there was frequently one at the opposite pole. In only one case was a black nucleus found near the pole when there was still a bud attached to it. The evidence gained from an examination of many slides containing cultures of this particular organism indicated that the nuclear material, increasing slightly in size, is gradually moved to the pole of the rod; the constriction process, by which it is finally separated from the parent cell as a gonidium, then begins.

The organisms in the other cultures were smaller and so were more difficult to examine. They showed some black cells and a very few gonidia.

While these methods of studying the morphologic changes of B. coli had yielded something of value for certain strains, they had, as a whole, failed because of the lack of uniformity of response by the cultures. For this reason it seemed advisable to try the influence of osmotic pressure on the bacterial cells.

Experiments with a Medium of High Solution Pressure.—The medium first employed for these tests was a standard beef agar containing from 2% to 10% of sodium chloride. The agar was selected in preference to the broth because in making a film for microscopic examination less foreign material is carried from the agar than from the broth.

All the strains grew well on the agar with a salt content up to 5%; above this amount the growth was less abundant, and at 8% all except 3 failed to grow. The majority of the strains showed a change in morphology.

When these preliminary tests were completed the cultures were plated on beef agar and duplicate beef agar slants were then inoculated from the typical colonies that appeared on the plates. The cultures were grown for one day at 37 C. and then inoculated into Clark and Lubs' synthetic phthalate-glucose phosphate solution for the determination of the Voges-Proskauer reaction and the methyl red test. The results were the same as those first obtained.

The pure cultures from this last plating were grown on both fresh and old salt agar and examined microscopically. The organisms from

old somewhat dry salt agar showed a slightly greater morphologic variation than the organisms on fresh salt mediums. In order that new mediums might be made more nearly comparable to the old dry one, which had given the best results, the amount of agar in them was increased to 2.5%. The mediums thus prepared showed no material change in P_H value from the standard one which gave a reading of 6.9 against that of 6.8 for those containing 5, 6 and 7% salt.

When grown on the beef agar containing 2.5 agar and 6% salt the cultures produced two characteristic types of growth. One gave a scanty to moderate filiform, raised, glistening, smooth, opaque, grayish white, slimy growth; the other gave a very scant filiform, flat, dull, granular, translucent, colorless, brittle growth. The 3 organisms that grew on agar containing 8% salt, belonged to the former group. A heavy inoculum on the salt agar slant usually produced a growth; a light one frequently failed to do so. Growth from the heavy inoculum started where the smear was thickest and the thin portion appeared to dry out. From this evidence it seems as if mass action may play a part in enabling the organisms to grow on this medium. There is also a possibility that growth may be due to the multiplication of the resistant organisms described by Hort.[13]

With this preliminary statement about the growth of B. coli on salted beef agar the morphologic variation induced by this medium may now be considered.

Results of Microscopic Examination. — Before considering the results of the microscopic examination of the strains, as shown by the accompanying photomicrographs, it must be clearly understood that the organisms in a culture do not all produce the same morphologic type on a medium. There is usually considerable variation in a single culture and also in repeated cultures as four transfers of the strains from beef agar to salted agar have shown. Three of the strains were very stable, little or no variation being evident; the remaining strains produced morphologic types that have been grouped under the following headings: gonidia, buds, coccoidal types, branched types, segmentation and threads. A brief explanation of these terms will be given when necessary under the respective headings.

Gonidia: Gonidia are the small, usually actively motile spherical or oval organisms which first appear on bacterial cells as buds, but which later, due possibly to their own active movements, become free. The organisms shown in the various fields on plate 2, under the designation

of gonidia, are in reality mature buds that are near the stage when they will be liberated as gonidia. The buds are shown in order to leave no doubt in the reader's mind concerning the identity of these minute cells.

A careful examination of all the B. coli on plain beef agar showed that on this medium only one culture produced gonidia in sufficient number to be readily found. The picture of this organism has already been referred to—field 24, plate 2. Gonidia may be seen at the poles of each of a number of the rods and threads and in some cases 3 or more of these reproductive units are at the pole of a mother cell. When the B. coli cultures were planted on salted beef agar slants they nearly all produced gonidia, but the count of the latter varied from very few to a moderate number, according to the culture. No strain, except 10, has ever produced them in the abundance shown in the field referred to. Fields 22 and 27 inclusive on plate 2, nearly all of different cultures, contain examples of gonidia and mature polar buds that are almost free. Above the thread with the bud at the upper pole in field 22, two slender rods appear to have produced a regenerative body which from its mode of production would be similar to a zygospore. Much importance must not be attached to this type, however, as it was the only example of the kind found. Fields 23a and 23b appear as normal B. coli-producing gonidia. In field 25a a branch is dividing from a mother cell; there are buds at both of the poles on the left of this organism. A single gonidium lies below the thread in field 25b; the thread has a mature bud at one pole.

Field 26 shows how differently cells in the same culture respond to the unfavorable environment. At the right of the picture a thread has divided into 4 rods of approximately normal size, while in the middle of the picture a cell has produced 4 short branches from which either rods have been set free by segmentation or gonidia liberated. A mature bud is about to be set free at the upper pole of this cell. Fields 28 to 30 inclusive, plate 2, show mature buds attached to the sides of threads. In the last picture of this group the thread to which the bud is attached is joined to the one below it by a short process. It was impossible to determine whether this was merely slime or whether it actually had some significance.

Buds: The term buds is here employed to designate the minute growths that push out from the bacterial cell; they first appear as a slight swelling which may grow into a branch or become a coccoidal cell attached to the parent. Only the beginning of bud formation is shown under this heading because pictures of mature buds have already

been employed to illustrate gonidial growth. Fields 31 to 42 inclusive, plate 2, contain examples of buds in different stages of growth. Nos. 31, 32a and 33a show the bud as a slight swelling in the parent rod; 33b shows a larger growth while in 34 and 35 the bud appears to be forming a coccoidal body at the extremity of the short stem as if it would free a spherical gonidium instead of growing to a rod and then undergoing segmentation. The latter process may have left the short branch remaining on the parent rod in 37, but the branch with the rod still attached may be clearly seen in 75, plate 4, where the branch at the right is dividing. In fields 32b and 42, plate 2, the bud has grown and produced a coccoidal body. A bud that has grown to a short rod and seems to be in the process of separating from the parent cell by sagittal segmentation may be found in the middle of field 80, plate 4 The appearance of some of the smaller rods in this field indicates that they too may have been produced in this manner.

Coccoidal Types: Small or large round living bodies of bacterial origin are here classified as coccoidal types. They may be either free or attached to a mother cell. The fields 22, 40 and 43a to 48b inclusive, plate B, and 49 to 68 inclusive, plate 3, all contain examples of coccoidal growth. The long threads produced by some of the organisms in the different cultures show the stimulatory effect of the salt and high osmotic pressure. When the nuclei start to grow in the parent cell, they frequently do so in the form that will best enable them to resist the high solution pressure, this form of course being coccoidal. Fields 22 and 40, plate 2; 49, 50, 51, 56a and 57 to 68, plate 3, all contain good examples of the growth of a nucleus within the mother cell. Field 42, plate 2, contains a much segmented thread; at the pole, where gonidia are usually liberated from the mother cell, the segment has become nearly coccoidal in form. While a nucleus is undoubtedly the source of larger growth in this polar cell as well as in 22, 40, 43a, 45, 47 and 48a, a nucleus is also very likely present in the fourth segment from the one previously mentioned in field 42. This cell is deeply stained like the polar coccoid and it also is larger than the other segments of the thread; again like the coccoid, but unlike the polar segment, it has maintained the rod-like form. In some threads the best types of coccoids are found nearer the middle of the cell (field 45, plate 2; 49, 57 and the upper thread in 60, plate 3).

The large oval, round or sickle shaped bodies found growing on threads, as in fields 50, 51, 58, 66 and 67, plate 3, seem in some cases to have a very soft cell wall as a number of them appear to have been distorted on the microscopic slide. In fields 50 and 51 these bodies

can be seen in all stages of growth The formation of one of these bodies in a thicker thread is shown in field 52; the cell in field 55b shows an equatorial growth much like it. Field 53 illustrates how scattered these centers of more vigorous growth are.

The lower cell in field 56b, plate 3, might be mistaken for a coccoidal body germinating, but the rod attached to it is the remnant of the mother cell which it would have lost when a little older, just as the older cell above it has lost the mother cell that was attached at the point that still protrudes from this otherwise spherical cell. The upper cell was near the stage when it would entirely disintegrate. In field 57, plate 3, the small rods with oval or spherical swellings look much like spore-bearing rods. These enlargements originated from the growth of a nucleus within the mother cell and the subsequent separation of a portion of this cell, as a small rod, by sagittal segmentation, as may be seen in process just a little above the middle of the field. Three free rods in the field have one pole either pointed or of indefinite outline, a characteristic of sagittal segmentation.

Field 59, plate 3, is interesting as showing the separation of a coccoidal body at one pole of a thread and a rod at the other. The long thread in field 60, plate 3, has a well defined coccoidal body at one pole and a slight swelling at the other as if a nucleus was also in process of growth at that point. The round cell in field 62a has two short processes extending toward the lower part of the picture. This was the only example found in the salt agar cultures that looked like germination. It may be that in some cases a nucleus after forming a coccoidal body, as this one has, is stimulated to produce a secondary growth from daughter nuclei but such instances must be very rare for in the examination of some thousands of slides, only one other was found, fields 11, 14 and 15, plate 1, on which were organisms that had this same appearance. In the latter case a number of such cells were present, while in the former only the one was found on the slide. The appearance of the cell in field 11 might also be due to the growth of a nucleus at the pole of the rod.

The cell in field 65, plate 3, is interesting because it illustrates well the growth of the nucleus in a bud to a large spherical cell. At the left of the organism is a short process at the pole of which is a small bud; at the right is a larger bud at the pole of a much longer process, while above this is a large coccoidal body at the pole of another branch.

Branched Types:. The term branched types is used to designate those bacterial cells that have a rod-like or thread-like outgrowth at an angle to the major axis of the parent cell.

Branched types exhibit considerable variation. Fields 65, plate 3; 69, 70, 72 and 76 inclusive, and 81 to 83, 85 and 86a and 86b, plate 4, show the differences in this kind of bacterial growth. Fields 72 and 73 were both found on the same slide, the former showing the beginning of a growth such as may be seen in the latter in more mature form. No indications were found in this culture that the branches separate from the parent thread as one is doing by sagittal segmentation in field 75. These branches seem to originate from the growth of a nucleus of a more resistant type in consequence of which they grow in the rod-like form despite the high solution pressure.

The appearance of the organisms in fields 81 to 83 and 85 to 86, plate 4, bear out the indications of a nuclear origin of this form of growth. Sometimes at the pole of the branch a daughter nucleus may grow and produce a coccoidal cell, fields 65, plate 3, and 79, plate 4; or rods, fields 86a and b. The nucleus at the end of the short process in field 34, plate 2, by growing to a cocoidal body or rod would present an appearance like the fields referred to. Fields 81 to 83, plate 4, may be further examples of this secondary growth in which the daughter cell is separating from the branch by sagittal segmentation. The forms in fields 10 and 13, plate 1; 81 to 84 and 86a and b, plate 4, will have to be studied in the hanging drop as there is a possibility that they may be types of conjunction.

Segmentation: Segmentation is the name given to the process of bacterial division when the cell wall becomes so softened that the daughter cells separate with either a pointed pole, field 80, plate 4 (to the right of the middle of the picture) or an ill-defined one, field 57, plate 3 (upper left corner).

In field 16 to 21 inclusive, plate 1, are shown examples of cell division on salt agar. Fields 16, 17 and 18 are the types of sagittal segmentation ordinarily seen. The cells in the remaining 3 pictures have the sharper lines of division characteristic of multiplication by fission. The coccoidal cell in the lower right hand corner of field 21 is dividing across the middle, while in the upper left hand corner the coccoidal body is beginning to separate from the parent rod. This group of pictures shows that although much changed morphologically, the fission habit of the species persists. It should be noted that the 2 kinds of division may be found in the same culture for fields 18 to 21 inclusive, were all taken from the same slide.

Threads: In addition to the morphologic types that have been described, many of the strains produce long threads. Pictures of these may be found in the various fields. Examples of some of the very long ones are shown in fields 71 and 78, plate 4. Such organisms have

given no indications of gonidium production, but they do divide into rods and threads; the thread in field 71 has divided near the middle into two parts.

In addition to the morphologic types shown under the group headings, some cultures produced on salt agar large cells with flagella-like processes similar to those which Hort [13] found in cultures in 4% glucose broth; field 54, plate 3, contains a picture of such a cell.

In the preliminary work with various mediums one culture was found which produced on starch agar, rod-like organisms at right angles to the mother cell. Field 12, plate A, is the example of this type of growth. Just below the middle of the picture in this field is a double cross; the two growing at right angles to the primary cells cross them at the points of juncture of the three. In this case the secondary growths could originate from polar buds.

Fields 6, 7a and 7b, plate 1, are pictures of the same culture grown under different conditions. It was isolated from the soil by Löhnis and identified as Bact. pneumoniae. Its pathogenicity has not been tested. The pictures are shown merely to illustrate the wide variation in morphology of a particular strain at different temperatures and on different mediums.

PHYSIOLOGIC REACTIONS

The effect of salt on the physiologic reactions of the bacteria was tested by planting organisms that had grown on salted beef agar in mediums with and without 5% salt and incubating them with similar mediums inoculated with organisms grown on standard beef agar.

The first test was made with cultures that had been transferred on two succeeding days to standard beef agar and then to agar containing approximately 2.5% agar and 6% salt. After one day's growth at 35 C. these cultures were inoculated into standard glucose broth and similar broth containing 5% NaCl, into brom-cresol purple milk and similar milk containing ½% NaCl and also into Clark and Lubs' synthetic phthalate medium, both plain and with the addition of 5% NaCl. After one day's growth at 35 C. it was found that among the strains from standard beef agar 8 had produced more gas, 7 the same amount and 7 less gas than the strains from the salted medium. When this test was made the total number of cultures had been reduced by an accident to 22.

The inoculums from standard beef agar produced in one day only from one quarter to one tenth as much gas in the salted glucose broth as in the standard one. The inoculums from salted beef agar showed a good growth in the salted broth, but only five produced gas. In each case only a small bubble was formed.

After 2 days' incubation the only tubes showing any marked difference in gas content were those containing salted glucose broth and inoculated from salted beef agar. These tubes contained between 10-20% gas. Even after 4 days they were, in general, slightly lower in gas content than the check tubes. All except 2 cultures, 17 and 26, rendered the brom-cresol-purple milk with and without salt acid in one day. These 2 cultures were the only ones that failed in 3 days to coagulate the standard milk whether the inoculum was taken from either standard or salted agar. In some instances it was necessary to employ heat to bring about coagulation. Only 3 cultures from standard beef agar coagulated the milk containing 0.5% salt in 4 days; two of these cultures also coagulated it when the inoculum was taken from salted beef agar. After 8 days' incubation the same 11 cultures from both inoculums coagulated the salted milk. The application of heat brought about the coagulation of the others.

Two sets of Clark and Lubs' synthetic mediums were tested, one of the regular formula inoculated with cultures from standard beef agar and the other containing 5% salt inoculated with cultures from salted beef agar. The salted medium showed little or no growth until the third day at 35 C. All tubes were tested on the fifth day for the Voges-Proskauer and the methyl red reactions. In the test for the Voges-Proskauer reaction only 2 cultures, 22 and 23, gave a positive result in the plain broth, and even these were negative in the salted one. Half of the cultures produced the same P_H concentration in broth medium and half produced a P_H from 0.3 to 0.7 less in the salted one.

DISCUSSION

It has long been recognized that in order to obtain duplicate or nearly duplicate results in tests with bacteria it is necessary to use mediums as nearly the same as our chemical methods will permit. This recognition of the influence of environment on the physiologic activity of bacteria is in accord with the general knowledge that all life responds to its environment. Some forms of life respond to change, both morphologically and physiologically, more than others, and not only different strains of the same species but often individuals of the same strain show marked variation. Since these morphologic changes are known to exist among other living things, it is not surprising that there is some morphologic response among the bacteria. As far as was observed with B. coli, however, it is only under extreme conditions of environment, such as on mediums containing 6% salt or, according to other investigators, 4% glucose and 0.2% malachite green, that there is a morphologic response which is sufficiently great to be easily recognized.

The extreme morphologic changes are produced by a selective process, the higher salt concentration tending to kill the weaker forms. Even the organisms that grew on the salted agar the first time showed weakened vitality with succeeding transfers. In order to maintain life, in addition to the usual method of multiplication by fission, they liberate gonidia at the poles or the sides of the rods or threads. The present work has shown definitely that chromatin material is concentrated within the rod into a nucleus and that this nucleus after growing slightly may, as a gonidium, separate from the parent cell and grow independently. This, however, is the exceptional case; the nuclei usually grow while still connected with the mother cell. On standard medium they remain within the mother cell, or there may be a few cells in each culture which produce the nuclei and liberate them as gonidia from the pole of the rod or more rarely from the side of the cell.

On a medium containing 6 or 7% salt and about 2.5% agar the protoplasm seems to be greatly stimulated. The nuclei also respond to this stimulus sometimes by a growth through the side of the mother cell. In some cases in which the sheath of the cell is weak the nucleus in growing retains a spherical shape as a protection against the osmotic pressure. The nucleus in response to the stimulus may start growth anywhere in the axis of the mother cell and produce a spherical body. The coccoidal forms which have only been found on mediums of high osmotic pressure grow until they are spent and begin to disintegrate when they may be found on the microscopic slide as shadow forms. A few that are more resistant retain the power, even in the coccoidal form, to multiply by fission; in some instances division takes place in the diameter of the sphere at right angles to the axis of the parent cell, in others the division takes place at the circumference of the coccoidal body. This evidence shows that the normal processes of the organisms are not destroyed; certain of them only are accelerated, i. e., growth and gonidia production.

The gonidia in growing form either rods or coccoids. The basis for this difference is not known, but it is presumed that those growing in the rod form are more resistant. It may be that the permeability of the cell sheath is a determining factor, for, like Wilson,[7] the author found that coccoidal forms were more plentiful in a medium inoculated from an old culture. In this case the gonidia would get very little protection from the selective permeability of the cell wall.

Some cultures on the salt agar produce nearly all large rods, others short threads and still others exceedingly long threads. A microscopic field generally shows a majority of one kind with some of the others

present. The difference in kinds of morphology produced by the organisms on the salted beef agar may depend on the fundamental quality that has given so much trouble in all bacteriologic work, namely, the inherent differences that exist in strains of the same species, due to their weakness or strength in a particular environment.

The gonidia are the units of reproduction and so apparently carry the inherited characteristics of the strain. The rod-like forms may divide or give off buds but, as they get old, the protoplasm to perpetuate the species forms nuclei which in the old cell of B. coli on standard mediums are referred to as polar granules. In a fresh medium these granules swell and meet in the middle of the old sheath. Whether or not they will grow without a sheath is a question which is unsettled for the proof to be gained by cutting the cell wall and observing the response of the nucleus thus exposed has not yet been attempted.

Organisms from a salted medium grew as fast as those from standard beef agar in milk with or without salt and in standard glucose broth with or without salt, but in the salted broth the organisms from standard agar showed retarded power to form gas while those from salted agar formed no gas in one day. From the fact that there appeared to be little or no growth in the Clark and Lubs' salted medium for the first two days, while the broths were cloudy and the milk acid, it seems that the odd morphologic forms and the gonidia recover their normal vitality quicker in the presence of complex organic buffers, like peptone or casein, than in the presence of inorganic ones. This is true even when the solution pressure is high enough to retard free growth.

The discovery of the importance of gonidia in propagating the species under certain conditions introduces a new factor that must be considered in any question dealing with bacterial multiplication. This is especially true in problems of pathology in which the gonidia as filtrable viruses may invade a host that the parent cell could not enter.

The results of other investigators of B. coli as here reviewed agree with those of the present report. It is accordingly evident that when placed under a variety of conditions different strains of this species have given an index of the extent of their life history and very likely that of some other non-spore-formers as well. It was found that the normal rod may be stimulated to produce threads or coccoidal forms. Gonidia may be liberated from these cells and become large organisms. If the nucleus grows in the parent cell, it may produce a branch or a globular body. The branch, like the parent, may produce gonidia or rods. On standard mediums the few gonidia produced grow to normal rods; the other morphologic types are rare.

SUMMARY

This investigation was made with strains of B. coli, B. aerogenes and four unidentified cultures. The conclusions drawn are considered as applying only to B. coli, although they may be equally true of many non-spore-forming species.

The cultures employed, in general, were very stable, but as a rule one or two different strains would show some variation in morphology on all the special mediums, like egg starch agar, etc.

When grown on standard beef agar, B. coli multiply almost entirely by fission but some produce gonidia. The number of the latter is usually small, varying with the strain. One culture was found which produced gonidia in abundance.

The gonidia when liberated on standard mediums grow to rods.

When transplanted to a medium of high osmotic pressure many rods die. The resultant growth is due either to the mass action of the organisms or to the presence of more resistant cells in the culture.

The vitality of a culture is reduced by repeatedly transferring to mediums of high solution pressure. All degrees of sensitiveness to this environment have been found.

Some rods and threads under special stimulus produce coccoidal bodies which arise from the growth of a nucleus within the parent cell. This type of growth was obtained on a medium of high solution pressure, i. e., 6% salt and 2.5% agar in the standard beef agar.

These coccoidal growths may separate from the parent cell by the division characteristic of cells with either firm or soft walls. The division is accordingly sharply defined, as typically occurs in fission, or it may be drawn out as in the case of sagittal segmentation. Both kinds of division are found in the same culture.

The coccoidal cells may liberate small cells by sagittal segmentation.

The large free coccoidal bodies become shadow forms and disintegrate if left on the medium which produced them.

A rod-like growth may originate within a mother cell and extend through the side wall of the parent.

Odd shaped cells are usually found in cultures grown on mediums of high solution pressure.

Those cultures that readily respond morphologically to a change in environment show a tendency on rich nitrogenous mediums, like egg agar or egg starch agar, to form small coccoidal bodies at 37 C. and rods, larger than those on standard mediums, at 17 C. The great majority of the organisms tested in this work showed only slight variation under these conditions.

The different morphologic types quickly revert to the normal laboratory type of B. coli when planted on a standard medium from one that has caused variation.

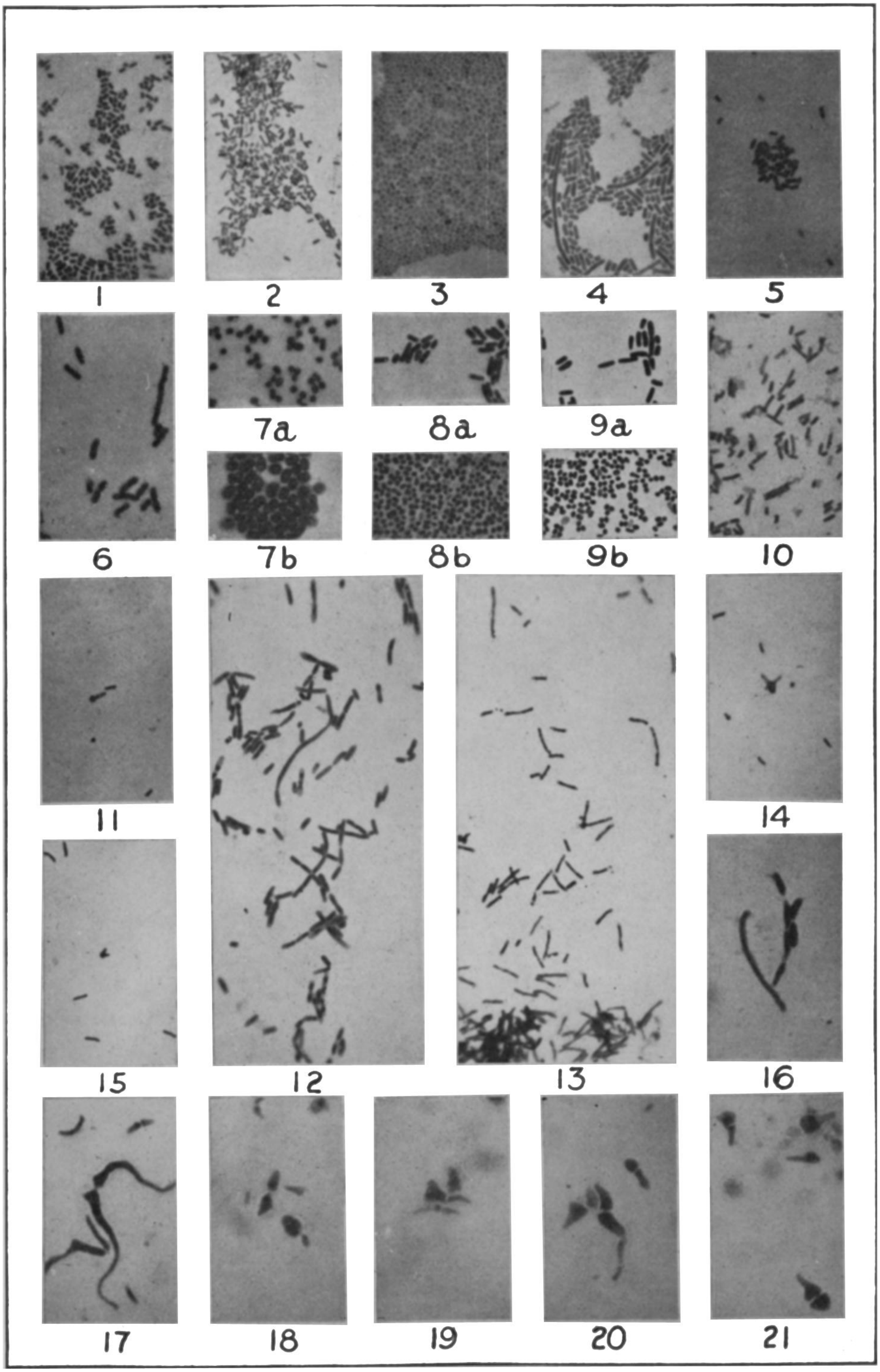
In mediums of high solution pressure all B. coli retained their physiologic activity though one or more functions of the strains were much suppressed. Gas formation was greatly retarded in all cultures and growth was delayed in Clark and Lubs' synthetic medium; the latter effect was very likely due to the absence of an organic buffer in this solution.

Explanation of Plate 1

Plate 1.—Magnification in all fields × 1000. All films stained with cold aqueous fuchsin.

1 Culture 2, beef agar, 2 days at 28 C.
2 Culture 13, beef agar, 2 days at 28 C.
3 Culture 11a, beef agar, 2 days at 28 C.
4 Culture 10, beef agar, 2 days at 28 C.
5 Culture 4, beef agar, 1 day at 37 C.
6 Bact. pneumoniae, beef agar, 1 day at 37 C.
7a Bact. pneumoniae, egg agar, 1 day at 19 C.
7b Bact. pneumoniae, starch agar, 1 day at 19 C.
8a Culture 14, egg starch agar, 1 day at 17 C.
8b Culture 14, egg starch agar, 1 day at 37 C.
9a Culture 27, egg starch agar, 17 day at 37 C.
9b Culture 27, egg starch agar, 1 day at 17 C.
10 Culture 10, beef agar, 1 day at 28 C.
11 Culture 16, ammonium lactate broth, 1 day at 28 C.
12 Culture 24, starch agar, 1 day at 37 C.
13 Culture 10, ammonium citrate broth, 5 days at 28 C.
14 Culture 16, ammonium lactate broth, 1 day at 28 C.
15 Culture 16, ammonium lactate broth, 1 day at 28 C.
16 Culture 3, beef agar plus 8% NaCl, 2 days at 35 C.
17 Culture 18, beef agar plus 6% NaCl, 1 day at 37 C.
18 Culture 26, beef agar plus 6% NaCl, 1 day at 37 C.
19 Culture 26, beef agar plus 6% NaCl, 1 day at 37 C.
20 Culture 26, beef agar plus 6% NaCl, 1 day at 37 C.
21 Culture 26, beef agar plus 6% NaCl, 1 day at 37 C.

PLATE 1



Explanation of Plate 2

Plate 2.—Magnification in all fields × 1000. All films stained with cold aqueous fuchsin.

22 Culture 11a, beef agar plus 6% NaCl, 1 day at 35 C.

23a Culture 2, beef agar plus 7% NaCl, 1 day at 35 C.

23b Culture 6, beef agar plus 7% NaCl, 7 days at 35 C.

24 Culture 10, ammonium lactate agar, 1 day at 37 C.

25a Culture 3, beef agar plus 8% NaCl, 2 days at 35 C.

25b Culture 18, beef agar plus 6% NaCl, 1 day at 35 C.

26 Culture 27, beef agar plus 6% NaCl, 1 day at 35 C.

27 Culture 11a, beef agar plus 6% NaCl, 1 day at 35 C.

28 Culture 18, beef agar plus 6% NaCl, 1 day at 35 C.

29 Culture 18, beef agar plus 6% NaCl, 1 day at 35 C.

30 Culture 11, beef agar plus 6% NaCl, 3 days at 35 C.

31 Culture 14, beef agar plus 6% NaCl, 1 day at 35 C.

32a Culture 14, beef agar plus 6% NaCl, 1 day at 35 C.

32b Culture 6, beef agar plus 8% NaCl, 1 day at 35 C.

33a Culture 10, beef agar plus 6% NaCl, 1 day at 35 C.

33b Culture 25, beef agar plus 6% NaCl, 1 day at 35 C.

34 Culutre 20, beef agar plus 6% NaCl, 1 day at 35 C.

35 Culture 1, beef agar plus 6% NaCl, 1 day at 35 C.

36 Culture 27, beef agar plus 6% NaCl, 1 day at 35 C.

37 Culture 26, beef agar plus 6% NaCl, 1 day at 35 C.

38 Culture 1, beef agar plus 6% NaCl, 1 day at 35 C.

39a Culture 19, beef agar plus 7% NaCl, 3 days at 35 C.

39b Culture 25, beef agar plus 6% NaCl, 1 day at 35 C.

40 Culture 3, beef agar plus 8% NaCl, 2 days at 35 C.

41 Culture 11, beef agar plus 6% NaCl, 3 days at 35 C.

42 Culture 25, beef agar plus 1% NaCl, 1 day at 35 C.

43a Culture 6, beef agar plus 7% NaCl, 1 day at 35 C.
Inoculated from day old growth on 6% NaCl.

43b Culture 27, beef agar plus 6% NaCl, 1 day at 35 C.

44 Culture 11, beef agar plus 6% NaCl, 18 hours at 35 C.
Inoculum 15 days old.

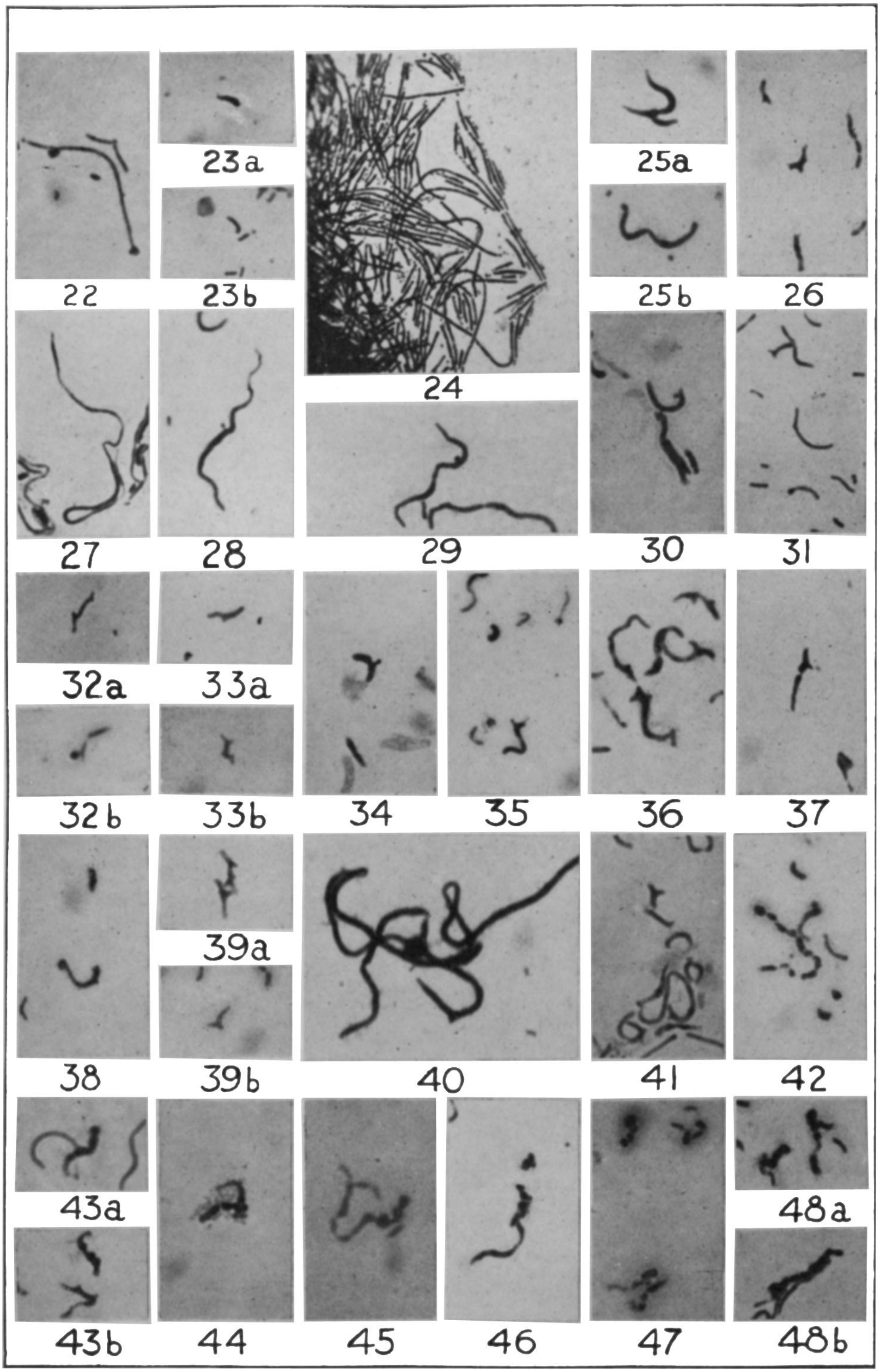
45 Culture 6, beef agar plus 7% NaCl, 1 day at 35 C.
Inoculated from 1 day growth on 6% NaCl agar.

46 Culture 27, beef agar plus 6% NaCl, 1 day at 35 C.

48a Culture 16, beef agar plus 6% NaCl, 1 day at 35 C.

48b Culture 11a, beef agar plus 6% NaCl, 1 day at 35 C.

PLATE 2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Explanation of Plate 3

Plate 3.—Magnification in all fields × 1000. All films stained with cold aqueous fuchsin.

49 Culture 1, beef agar plus 6% NaCl, 1 day at 35 C.

50 Culture 4, beef agar plus 7% NaCl, 1 day at 35 C.
Inoculated from 1 day growth on 6% NaCl agar.

51 Culture 10, beef agar plus 6% NaCl, 18 hours at 35 C.
Inoculated from 15 day growth on plain agar.

52 Culture 27, beef agar plus 6% NaCl, 1 day at 35 C.

53 Culture 1, beef agar plus 7% NaCl, 1 day at 35 C.

54 Culture 1, beef agar plus 7% NaCl, 1 day at 35 C.

55a Culture 4, beef agar plus 7% NaCl, 1 day at 35 C.
Inoculated from 1 day growth on 6% NaCl agar.

55b Culture 2, beef agar plus 6% NaCl, 1 day at 35 C.

56a Culture 4, beef agar plus 7% NaCl, 1 day at 35 C.
Inoculated from 1 day growth on 6% NaCl agar.

56b Culture 4, beef agar plus 7% NaCl, 7 days at 35 C.

57 Culture 20, beef agar plus 6% NaCl, 1 day at 35 C.

58 Culture 4, beef agar plus 7% NaCl, 1 day at 35 C.
Inoculated from 6% NaCl agar.

59 Culture 14, beef agar plus 6% NaCl, 1 day at 35 C.

60 Culture 19, beef agar plus 6% NaCl, 3 days at 35 C.

61 Culture 6, beef agar plus 8% NaCl, 1 day at 35 C.

62a Culture 25, beef agar plus 6% NaCl, 1 day at 35 C.

62b Culture 3, beef agar plus 6% NaCl, 2 days at 35 C.

63a Culture 27, beef agar plus 6% NaCl, 1 day at 35 C.

63b Culture 4, beef agar plus 7% NaCl, 7 days at 35 C.

64 Culture 2, beef agar plus 7% NaCl, 1 day at 35 C.

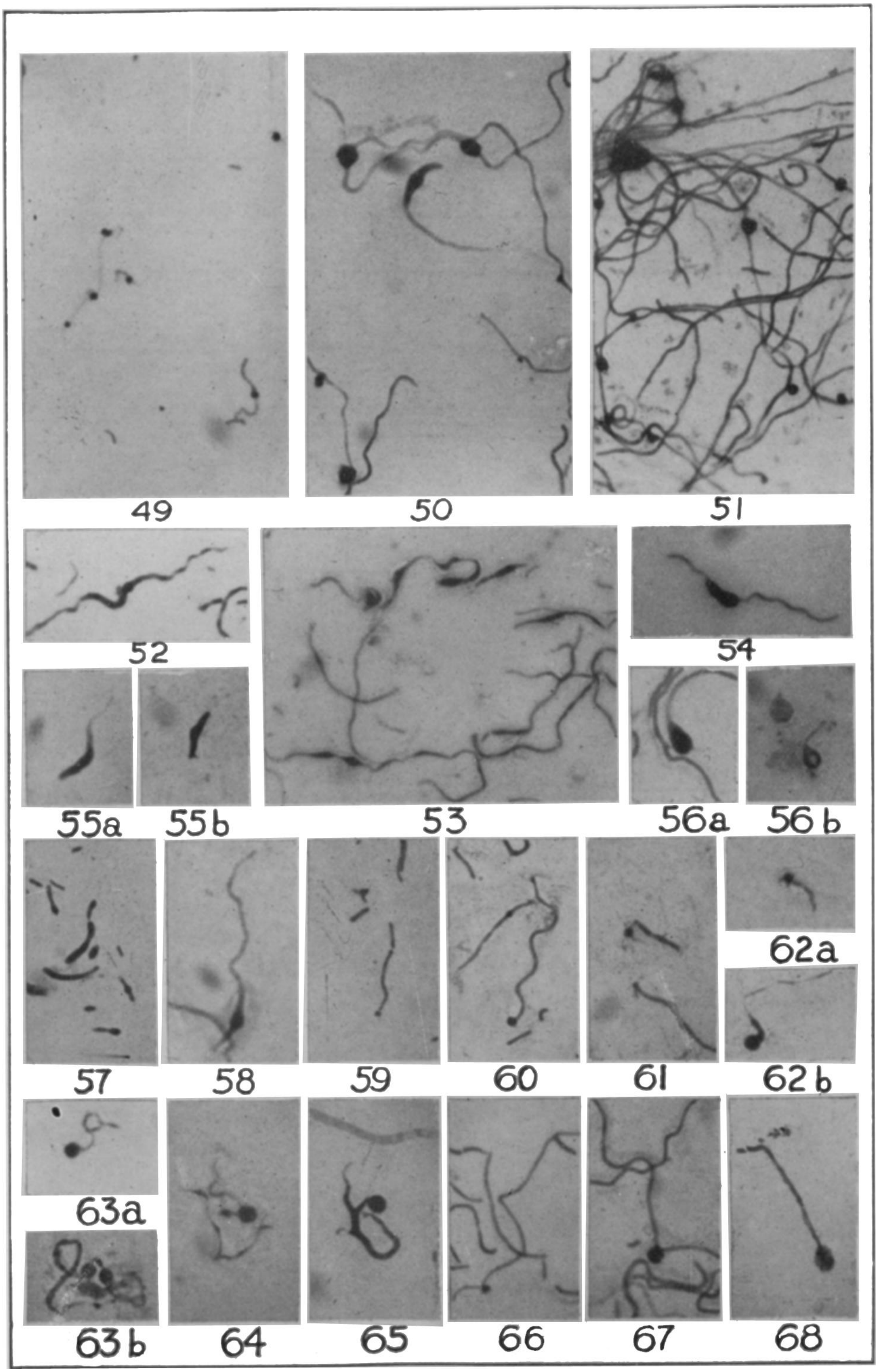
65 Culture 3, beef agar plus 8% NaCl, 2 days at 35 C.

66 Culture 14, beef agar plus 6% NaCl, 1 day at 35 C.

67 Culture 4, beef agar plus 7% NaCl, 1 day at 35 C.
Inoculated from 1 day growth on 6% NaCl agar.

68 Culture 3, beef agar plus 7% NaCl, 1 day at 35 C.

PLATE 3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Explanation of Plate 4

Plate 4.—Magnification in all fields × 1000. All films stained with cold aqueous fuchsin.

69 Culture 3, beef agar plus 7% NaCl, 1 day at 35 C.

70 Culture 10, beef agar plus 6% NaCl, 1 day at 35 C.

71 Culture 1, beef agar plus 7% NaCl, 1 day at 35 C.

72 Culture 4, beef agar plus 7% NaCl, 7 days at 35 C.

73 Culture 4, beef agar plus 7% NaCl, 7 days at 35 C.

74 Culture 10, beef agar plus 6% NaCl, 18 hours at 35 C. Inoculum 15 days on standard beef agar.

75 Culture 3, beef agar plus 8% NaCl, 2 days at 35 C.

76 Culture 11a, beef agar plus 6% NaCl, 3 days at 35 C.

77 Culture 27, beef agar plus 6% NaCl, 1 day at 35 C.

78 Culture 1, beef agar plus 7% NaCl, 1 day at 35 C.

79 Culture 3, beef agar plus 8% NaCl, 2 days at 35 C.

80 Culure 20, beef agar plus 6% NaCl, 1 day at 35 C.

81 Culture 26, beef agar plus 6% NaCl, 1 day at 35 C.

82 Culture 2, beef agar plus 7% NaCl, 1 day at 35 C.

83 Culture 11, beef agar plus 7% NaCl, 3 days at 35 C.

84 Culture 18, beef agar plus 7% NaCl, 1 day at 35 C.

85 Culture 2, beef agar plus 7% NaCl, 1 day at 35 C.

86a Culture 11, beef agar plus 6% NaCl, 3 days at 35 C.

86b Culture 6, beef agar plus 7% NaCl, 1 day at 35 C.

PLATE 4